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Original Articles

The molecular signature of AML mesenchymal stromal cells reveals candidate genes related to the leukemogenic process

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ABSTRACT

Acute myeloid leukemia (AML) is a heterogeneous disease characterized by myeloid precursor proliferation in the bone marrow, apoptosis reduction and differentiation arrest. Although there are several studies in this field, events related to disease initiation and progression remain unknown. The malignant transformation of hematopoietic stem cells (HSC) is thought to generate leukemic stem cells, and this transformation could be related to changes in mesenchymal stromal cell (hMSC) signaling. Thus, the aim of this work was to analyze the gene expression profile of hMSC from AML patients (hMSC-AML) compared to healthy donors hMSCs (hMSC-HD). The results showed a common molecular signature for all hMSC-AML. Other assays were performed with a large number of patients and the results confirmed a molecular signature that is capable of distinguishing hMSC-AML from hMSC-HD. Moreover, *CCL2* and *BMP4* genes encode secreted proteins that could affect HSCs. To verify whether these proteins are differentially expressed in AML patients, ELISA was performed with plasma samples. CCL2 and BMP4 proteins are differentially expressed in AML patients, indicating changes in hMSC-AML signaling. Altogether, hMSCs-AML signaling alterations could be an important factor in the leukemic transformation process.

Introduction

Acute myeloid leukemia (AML) is a heterogeneous clonal disorder of hematopoietic progenitor cells characterized by myeloid precursor proliferation and accumulation in the bone marrow, apoptosis reduction and dysfunctional differentiation [1].

The malignant alteration of hematopoietic stem cells leads to a loss of normal hematopoietic function, which, if left untreated, leads to death within weeks to months of its clinical presentation [2].

The French, American and British group of leukemia experts (FAB) divided acute myeloid leukemia into eight subtypes based on the type of cell from which the leukemia developed and based on the level of maturation. Currently, the World Health Organization (WHO) provides a classification system in which morphology, cytogenetic, molecular genetics and immunological markers are related [3].

The cellular and molecular basis for the heterogeneity of AML represents a fundamental problem that has interested cancer researchers. Although several studies have examined this problem, the events related to the AML initiation and progression remain unclear.

The malignant transformation of normal hematopoietic stem cells (HSCs) is believed to generate leukemic stem cells (LSCs). Similar to normal HSCs, LSCs are characterized by their self-renewal ability, repopulating potential and progeny cell production. LSCs have also been reported to express stem cell markers and to survive indefinitely upon serial transplantations in animal models [4].

Transforming events could occur in a HSC, and one of the consequences of this event is the inability of this stem cell to generate normal progenitors and mature blood cells, thus generating its malignant counterpart, the LSC. The similar cell surface phenotypes of LSCs and HSCs support the idea that primitive cells are the origin cells for AML regardless of the maturation state of leukemia blasts [5].

Lapidot and co-workers described the existence of LSCs for AML. The engraftment of human LSC preparations into xenogeneic transplantation models has been regarded as evidence of the presence of leukemia-initiating cells for human AML [6].

The concept that the tumor cells present stem cell properties implies that LSC arises as an intrinsic property of tumor biology and development. However, the surrounding microenvironment including fibroblasts, adipocytes, endothelial cells, and the extracellular matrix play important roles in cancer progression [7]. The direct

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contact among stem cells and their niches is essential in regulating asymmetric divisions and promoting stem cell renewal [6].

HSCs reside in two distinct niches: the endosteal and the vascular. Signals from cells in these niches provide the clues for quiescence, self-renewal, survival and fate specification of these cells. Recent studies have shown that spatially distinct vascular niches for quiescent and nonquiescent HSCs are present in the bone marrow [8]. Although LSCs have similar microenvironment, it has been reported its expansion at hypoxic bone marrow areas in endosteal niche [9], contrary to what happens with HSCs that in hypoxic conditions are maintained in a quiescent state. Thus, the malignant transformation that generates LSCs could be related to changes in some niche component signaling.

LSCs localize and associate with cells around the endosteum to form discrete niches. Other cell types, including mesenchymal stromal cells (MSCs), which contribute to this niche, play a major role in LSC biology [10].

It is suggested that genetic changes in bone marrow stromal cells are not trivial because they are able to differentiate into osteolineage progenitors (osteoblast precursors) and initiate a malignant process in the normal HSC endosteal niche [10].

In this context, the aim of this work was to analyze the profile of human mesenchymal stromal cells (hMSCs) in acute myeloid leukemia patients. To address this hypothesis, we used chip arrays to evaluate the gene expression profiles of hMSCs from AML patients (hMSC-AML) compared to the profiles of hMSCs from healthy donors (hMSC-HD). The results showed that a common molecular signature exists for all hMSCs-AML compared to hMSCs-HD and that some of the genes that were differentially expressed could be related to malignant transformation.

Materials and methods

Patient and donor samples

Bone marrow (BM)-derived samples were obtained from patients diagnosed with acute myeloid leukemia (without any treatment) and from healthy donors. The 33 samples obtained from AML patients were morphologically characterized by immunophenotype as shown in Table 1. BM aspirates from AML patients were obtained from 19 male patients (mean age: 36.9; age range: 14–78 years) and 14 female patients (mean age: 40.8; age range 9–70) years. These patients were stratified into four cohorts: chip array cohort (n = 7), unsupervised chip array cohort (n = 10), RT-qPCR cohort (n = 19) and ELISA cohort (n = 33) (Table 1). All samples were provided from different hospitals of Rio de Janeiro and other states in Brazil. As control, we used 13 samples obtained from healthy donors that were registered at the Bone Marrow Transplantation Unit, National Cancer Institute (Rio de Janeiro, Brazil). BM aspirates from healthy donors were obtained from 5 male donors (mean age: 40.3 years; age range 30–59 years) and 8 female donors (mean age: 40.4 years; age range: 32–47 years). All samples were obtained in accordance with the guidelines of the local Ethics Committee and the Helsinki Declaration. This study was approved by the National Cancer Institute Ethics Committee (no. 034/06) and all participants signed informed consent forms.

Isolation and culture of human BM-derived mesenchymal stromal cells (hMSCs)

Bone marrow samples from AML patients and healthy donors were centrifuged at 1200 rpm for 15 min. After the samples were centrifuged, plasma from each sample was collected, treated with 1 μL/mL protease inhibitor mix (GE Healthcare Life Sciences, PA, USA) and frozen at −70 °C.

The precipitates obtained after BM centrifugation were plated in non-coated 25-cm^2 polystyrene culture flasks (TPP, Switzerland) in low-glucose Dulbecco's modified Eagle's medium (DMEM-LG, Invitrogen™, CA, USA) supplemented with 10% fetal bovine serum (FBS, Hyclone, USA), 100 IU/mL penicillin, 100 μg/mL streptomycin (Invitrogen™, CA, USA), and 2 mM L-glutamine (Invitrogen™, CA, USA). The cells were cultured at 37 °C in a humidified atmosphere of 5% CO_2.

After the cells were cultured for one week, non-adherent cells were removed from the culture flask, and the medium was changed twice a week until the cul-

Table 1
List of AML patients that participated in this study.

Laboratory code	FAB subtype	%Blasts	Sex	Age	Chip array cohort AML samples	Unsupervised chip array cohort AML samples	RT-qPCR cohort AML samples	ELISA cohort AML samples
005/12	M4/M5	80%	Male	43	x	x	x	x
006/12	M3	85%	Male	68	x	x	x	x
007/12	M1/M2	75%	Female	9	x	x	x	x
009/12	M3	80%	Male	30				x
010/12	M1/M2	36%	Male	18	x	x		x
011/12	M1/M2	64%	Female	13			x	x
012/12	M1/M2	60%	Female	13	x	x	x	x
017/12	M0	40%	Female	62				x
002/13	M1	29%	Female	70				x
007/13	M2	23%	Male	14				x
008/13	M2	98%	Male	42	x	x	x	x
009/13	M2	56%	Male	30	x	x	x	x
010/13	M2	38%	Male	25			x	x
017/13	M3	71.80%	Female	21			x	x
021/13	M4/M5	60%	Female	22				x
025/13	M4/M5	75%	Male	46		x	x	x
028/13	M2	17%	Male	26			x	x
031/13	M2	70%	Female	61			x	x
036/13	M3	84%	Female	29		x	x	x
037/13	M4/M5	80%	Male	35		x	x	x
040/13	M2	84%	Male	28				x
041/13	LMA unclassified	56%	Male	45				x
042/13	LMA unclassified	90%	Male	78				x
043/13	M3	80%	Female	53				x
045/13	M3	75%	Male	31			x	x
047/13	M2	87%	Female	47				x
050/13	M2	25%	Male	28			x	x
051/13	M1	67%	Male	50			x	x
053/13	M1	90%	Male	32			x	x
054/13	LMA unclassified	60%	Male	32			x	x
056/13	M4/M5	90%	Female	60				x
059/13	M3	98%	Female	47				x
061/13	M4/M5	84%	Female	65				x

tured hMSCs reached 80% confluence. Then, hMSCs were removed from the plates by treatment with 0.05% trypsin (Invitrogen™, CA, USA) for 5 min at 37 °C and then replated in another culture flask at a density of 2000 cells/cm^2 (passage 1). When 80% confluence was obtained (approximately 7 culture days), the cells were trypsinized and replated in another fresh culture flask (passage 2). These processes were repeated up to passage 3, when hMSCs were used for all experiments.

Confirmation of hMSCs

To confirm the multipotentiality of hMSCs used in this work, experiments were performed in accordance with the minimal criteria for defining multipotent mesenchymal stromal cells as defined by the International Society for Cellular Therapy [11].

The cultured plastic-adherent cells expressing the markers CD73 (#561254), CD90 (#561970) and CD105 (#561443) but not expressing the lineage commitment markers CD14 (# 347493), CD19 (#340951), CD34 (#348057), CD45 (#347463) and HLA-DR (#349528) (BD Biosciences, CA, USA) were able to differentiate into adipocytes and osteoblasts.

The ability of hMSC cultures to differentiate into adipocytes and osteoblasts was also tested at passage 3. To induce adipogenic differentiation, hMSCs were cultured with adipocyte differentiation basal medium (Gibco, CA, USA), and Oil-red-O (Sigma-Aldrich, MO, USA) was used to detect lipid accumulation. Osteogenic differentiation was induced using osteocyte differentiation basal medium (Gibco, CA, USA). Osteoblasts were identified by Alizarin Red S (Isofar, Brazil) staining.

Proliferation assay

Cell proliferation was measured using a 4-[3-(4-Iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1.3-benzene disulfonate (WST-1) assay (Roche, Germany). For this assay, 6.2×10^2 cells were plated in 96-well microplates. After 48, 96 and 144 h, 3 μL of WST-1 was added to each well, and the microplates were incubated at 37 °C for an additional 3 h. Then, the plates were read on a microplate reader (Bio-Rad, model 550, CA, USA) at 450 nm with a reference wavelength at 630 nm. Data analyses and graphical representations were performed using GraphPad Prism™ software (GraphPad Software Inc., CA, USA). All experiments were performed in triplicate.

Cell cycle assay

The cell cycle was analyzed using propidium iodide (PI; Sigma-Aldrich, MO, USA) as described by Nicoletti et al. [12]. The cell cycle was blocked by reducing FBS to 0.1% for 24 h, and then the concentration of FBS was returned to 10%. Three days later, the cells were harvested for cell cycle analysis. First, the cells were washed and fixed overnight in cold ethanol (70%). Then, the fixed cells were washed and reconstituted in 250 μL of buffer (0.1% NP40, 0.2 mg/mL RNase, and 0.2 mg/mL PI) and incubated for 30 min at 4 °C. Ten thousand events were collected from each sample using a FACSCalibur flow cytometer (BD Biosciences, CA, USA). All experiments were performed in triplicate. The data were analyzed using CellQuest software (BD Biosciences, CA, USA).

Expression chip array data analysis

Total RNA from hMSC cultures in passage 3 was obtained using TRIzol reagent (Invitrogen, CA, USA) and purified using an RNeasy Mini Kit (Qiagen, CA, USA) according to the manufacturer's instructions. One hundred nanograms (100 ng) of total RNA was used to synthesize biotinylated cRNA using a GeneChip Whole Transcription (WT) Sense Target Labeling Assay Kit (Affymetrix, CA, USA). Then, the biotinylated cRNA was hybridized to GeneChip Human Gene 1.0 ST Arrays (Affymetrix, CA, USA), washed and stained according to the manufacturer's protocols. The GeneChip arrays were scanned using a GeneChip® Scanner 3000. Affymetrix Expression Console software version 1.0 was used to create summarized expression values (CHP-files), and the robust multichip analysis (RMA) algorithm was applied. The data were analyzed using Partek® software (http://www.partek.com) [13], with differentially expressed genes with a ≥2-fold change used as criteria to define overexpression or downregulation. The pathway analysis and related processes were obtained using MetaCore™ software (http://thomsonreuters.com/metacore/).

Quantitative PCR (RT-qPCR)

RT-qPCR analyses were performed using two micrograms of mRNA treated with amplification grade DNase I (Invitrogen, CA, USA) and reverse transcribed with Superscript III Reverse transcriptase® (Invitrogen, CA, USA). Each reaction was performed with 5 μL of SYBR Green PCR Master Mix® (Applied Biosystems, CA, USA), 2.5 μL of cDNA (10 ng of cDNA) and 2 μM each primer. The quantitative determination of mRNA levels was performed using Rotor-Gene 6000 Series software (Corbett, Australia). The reactions were performed in a Rotor-Gene 6000 thermocycler (Corbett, Australia) using the following program: 95 °C for 5 min, followed by 45 cycles at 95 °C for 15 s, with a final extension at 62 °C for 40 s. Dissociation curve analysis was used to demonstrate equal amplification efficiency of a specific PCR product for all primers used in this study; all primers demonstrated equal amplification efficiency and specific PCR products through dissociation curve analysis. The determination of fold expression change was calculated using the DDCt method according to Livak and Schmittgen [14]. Expression levels were estimated in triplicate, and *B2M* and *GAPDH* were used as normalization genes. The following primers were used: *GAPDH* Fw (5′-GTCAACGGATTTGGTCGTATTG-3′) and Rev (5′-TGGAAGATGGTGATGGGATTT-3′), *B2M* Fw (5′-ATGAGTATGCCTGCCGTGTGA-3′) and Rev 5′-CGGCATCTTCAAACCTCCATG-3′), *CCL2* Fw (5′-CCCAGTCACCTGCTGTTAT-3′) and Rev (5′-GGAGTTTGGGTTTGCTTGTC-3′), *SPON2* Fw (5′-GGAAGAACCAGTACGTCAGTAAC-3′) and Rev (5′-GCACCACAAA CGAGACCA-3′), *MMP16* Fw (5′-TATTCGCCGTGCCTTTGAT-3′) and Rev (5′-CCACATC ACGTTTGCCATTT-3′), *CLDN1* Fw (5′-TCTTTGACTCCTTGCTGAATCT-3′) and Rev (5′-GCCAGACCTGCAAGAAGAAATA-3′), *BMP4* Fw (5′-CCATGATTCCTGGTAACCGA-3′) and Rev (5′-CCTGAATCTCGGCGACTT-3′), and *SSP1* Fw (5′-GCTAAACCCTGACCCATCTC-3′) and Rev (5′-CTACATCATCAGAGTCGTTCGAG-3′). The results were compared using the Mann–Whitney test. Statistical analysis and graphical representations were performed using GraphPad Prism™ software (GraphPad Software Inc., CA, USA).

ELISA

To perform ELISA, we used the bone marrow plasma samples that were collected previously from AML patients and from healthy donors and stocked at −70 °C. The levels of BMP4, SSP1 (Sigma-Aldrich, MO, USA) and CCL2 (Peprotech, NJ, USA) were determined in triplicate using specific ELISA kits according to the manufacturers' instructions. Statistical analysis and graphical representations were performed using GraphPad Prism™ software (GraphPad Software Inc., CA, USA).

Statistical analysis

All experiments were carried out in triplicate and the data were expressed as the mean ± standard error of the mean. The results were compared using an unpaired Mann–Whitney test and a p-value <0.05 was considered as statistically significant ($^*p < 0.05$, $^{**}p < 0.01$). Statistical analysis and graphical representations were performed using GraphPad Prism™ software (GraphPad Software Inc., CA, USA).

Results

In vitro *differentiation potential of hMSC cultures*

To determine whether the expanded hMSC cultures maintained multipotency differentiation characteristics, we tested hMSCs from AML patients and from healthy donors at passages 3 for differentiation into adipogenic and osteogenic cells. hMSCs cultured in adipogenic medium for 3 weeks showed lipid droplets characteristic of adipogenic cells by Oil Red O staining (Fig. 1C, D). Osteogenic differentiation was demonstrated by calcium deposition, which was stained by Alizarin Red S (Fig. 1E, F). Undifferentiated hMSCs from AML patients and from healthy donors were used as controls (Fig. 1A, B). Our results showed that hMSC cultures from both AML patients and healthy donors were able to differentiate into adipogenic and osteogenic cells that maintained their multipotency capacity. However, interestingly, while adipogenic differentiation did not present any differences between both types of hMSC cultures, osteogenic differentiation presented some differences when comparing hMSCs from patients and from healthy donors. As shown in Fig. 1E and F, while both cultures were able to differentiate into osteogenic cells, the hMSC cultures from AML patients presented a decreased potential for osteogenic differentiation.

hMSCs from AML patients displayed conserved proliferation and cell cycle profiles

To verify whether the hMSC cultures from AML patients presented changes in their proliferation potential and had cell cycle arrest, we performed WST and flow cytometry assays, respectively, and compared the results with those of hMSC cultures from healthy donors. As observed in Fig. 2A and B, the proliferation and

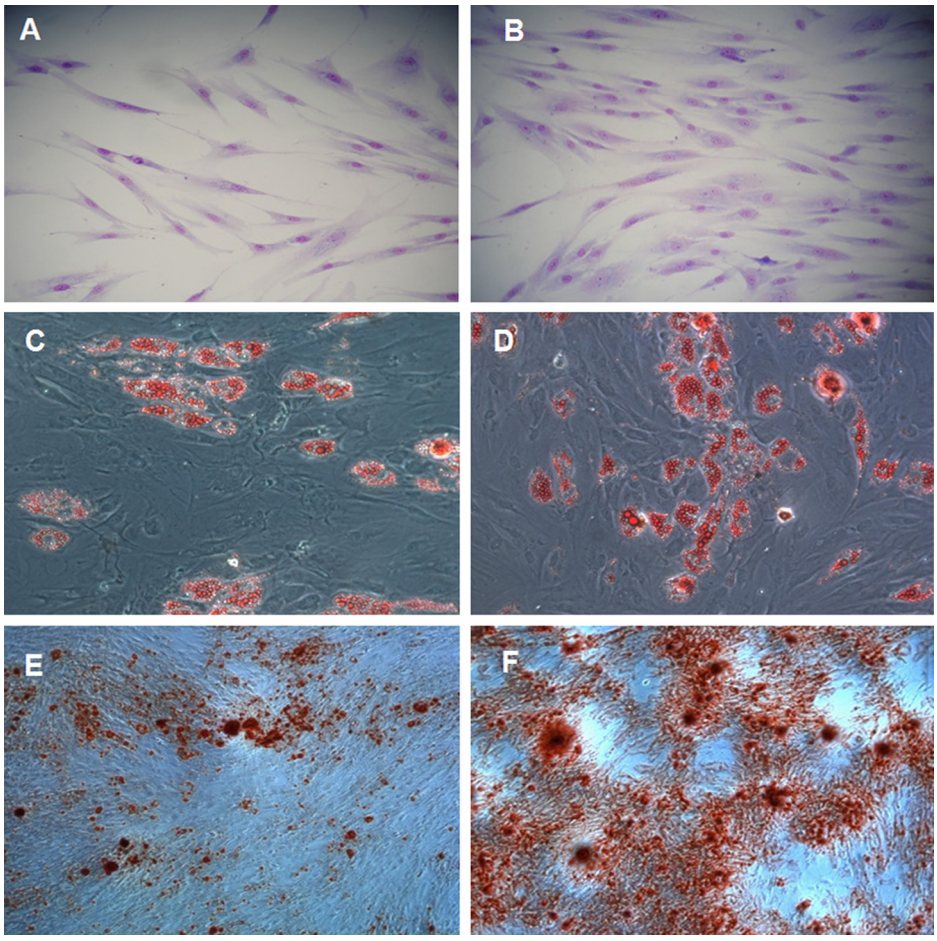

Fig. 1. hMSC multipotency capacity. (A and B) Undifferentiated hMSCs from healthy donors (HD) and AML patients, respectively (200× magnification). (C and D) Adipogenic differentiation of hMSCs from HD and AML patients, respectively. Accumulation of neutral lipid vacuoles stained with oil red O indicate differentiation (200× magnification). (E and F) Osteogenic differentiation of hMSCs from HD and AML patients, respectively. Calcium deposition stained with alizarin red indicates differentiation (50× magnification).

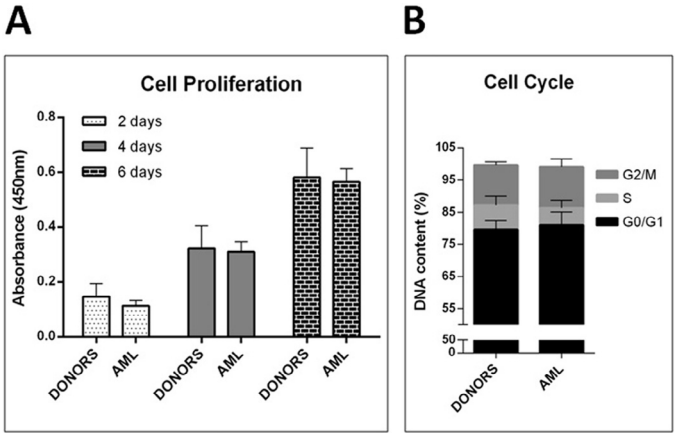

Fig. 2. Cell proliferation and cell cycle analysis. (A) Cell proliferation profiles of hMSC-AML and hMSC-HD were determined after 48, 96 and 144 h of culture by WST-1 assay. (B) Using the same samples, flow cytometry was used to analyze the cell cycle. The results did not differ among all cultures.

cell cycle profiles were identical for all hMSC cultures; no significant alterations were observed. These results indicate that hMSC cultures conserved their proliferation capacity in the culture instead of that of the disease.

Fifty-five genes define a hMSC-AML molecular profile

Although hMSCs from AML patients conserved their proliferation profile, the molecular pattern of these cells could differ from that of hMSCs from healthy donors. Thus, we determined the global gene expression pattern for hMSC cultures from AML patients and compared it with the global gene expression pattern for hMSC cultures from healthy donors. We performed a comparative transcriptome analysis using an expression chip array assay.

In this assay, 7 samples from different AML subtypes were used and compared with two pools of healthy donors. After the 3rd passage, total RNA was obtained from each hMSC culture, processed, hybridized to GeneChip Human Gene 1.0 ST Arrays (Affymetrix, CA, USA), washed and stained according to the manufacturer's protocols. Using a ≥2-fold change as a cut-off to define overexpression or downregulation, fifty-five genes were found to be differentially expressed in all chip array assays. The hierarchical clustering of these differentially expressed genes shown in Fig. 3 suggests that a common molecular signature exists for all MSCs from AML patients compared to that for MSCs from healthy donors. Notably, 11 of these 55 genes were overexpressed in hMSC-AML cultures, while 44 of these genes were downregulated, indicating a global gene expression decrease in hMSC cultures from AML patients (Table 2).

In silico analysis of these results using MetaCore™ software showed that the genes found altered in hMSCs from AML patients

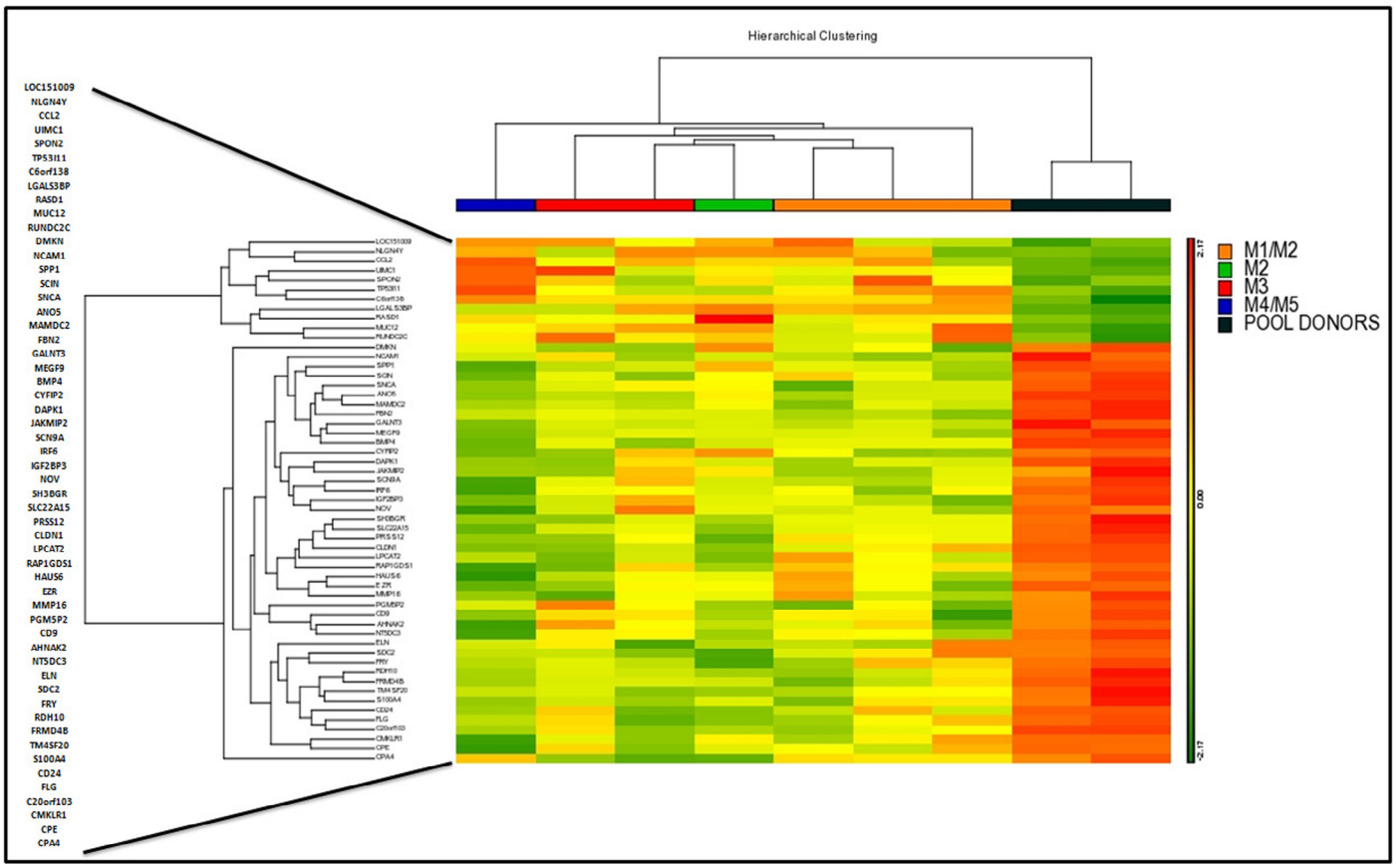

Fig. 3. Hierarchical clustering of the 55 differentially expressed genes identified by chip array assay. The results showed a common molecular signature for all hMSCs from AML patients compared to hMSCs from healthy donors.

function in important signaling pathways during normal hematopoiesis. These genes are involved primarily in processes of cellular adhesion, the Osteopontin (SSP1) pathway and the WNT pathway, among other functions. Both SSP1 and WNT are important signaling pathways related to HSC maintenance and differentiation, respectively [15,16].

RT-qPCR and unsupervised chip array assay confirmed the molecular signature of hMSC-AML cultures

To confirm the obtained chip array results, quantitative PCR (RT-qPCR) of some overexpressed and downregulated genes were performed. In this case, the analysis was performed with a larger number of patients with different subtypes (n = 19) and with healthy donors (n = 13). The overexpressed genes, *i.e.*, *CCL2* and *SPON2*, and the downregulated genes, *i.e.*, *MMP16*, *BMP4*, *CLDN1* and *SSP1*, in hMSC-AML cultures were used in this analysis. These genes were chosen because all of them had been previously described as related to the microenvironment, HSC or AML. *CCL2* and *MMP16* were selected because they are related to the bone marrow microenvironment and to AML [17,18]. *BMP4* and *SSP1* genes are important for the development and maintenance of HSCs [15,19], and *SPOND2* and *CLDN1* were chosen because they are related to neoplasia [20,21]. The RT-qPCR results presented in Fig. 4 confirmed the obtained chip array assay results.

Furthermore, we performed another chip array assay using 3 additional AML patient samples. At this time, an unsupervised analysis of the 55 differentially expressed genes was performed with these 3 new hMSC samples from AML patients and with the other 7 samples used previously to verify whether the samples could group into clusters that separate AML patients from healthy donors. As shown in Fig. 5, these 55 genes were able to distinguish the hMSC-AML cultures from hMSC-HD cultures.

Secreted proteins from hMSC-AML cultures could be related to malignant transformation

Changes in MSC signaling could be related to the malignant transformation of HSCs to LSCs. Some of the genes identified by the chip array assay are secreted proteins that could affect HSCs. To analyze whether these secreted proteins are differentially expressed in AML patients, we performed ELISA with the bone marrow plasma samples from 33 AML patients and from 11 healthy donors. The secreted proteins selected were CCL2, SSP1 and BMP4 (CCL2 is overexpressed and the others are downregulated in AML patients). The results confirmed the increase in CCL2 protein levels and the decrease in BMP4 protein levels in AML patient bone marrow plasma samples, suggesting that MSC signaling differs between hMSC cultures from healthy donors and AML patients (Fig. 6A, B). However, no significant changes were observed for SSP1 protein levels (Fig. 6C). All of these proteins have been described as important components produced by the hematopoietic microenvironment that regulates both HSC number and function. Thus, this modified signaling could be related to the malignant transformation of HSCs to LSCs.

Discussion

The BM microenvironment is composed of hematopoietic stem cells (HSCs) and several nonhematopoietic cells types including osteoblasts and osteoclasts, sinusoidal endothelial cells, fibroblasts, reticular cells, mesenchymal stromal cells (MSCs), perivascular stromal cells, immune cells, and several others which may play a

Table 2
List of the 55 differentially expressed genes identified by chip array assay.

Gene symbol	Gene_assignment	RefSeq	Fold-change	AML vs POOL DONORS
NLGN4Y	NR_028319 // NLGN4Y // neuroligin 4, Y-linked // Yq11.221 // 22829 /// NM_001164	NR_028319	5.55	AML up vs POOL DONORS
SPON2	NM_012445 // SPON2 // spondin 2, extracellular matrix protein // 4p16.3 // 10417	NM_012445	2.62	AML up vs POOL DONORS
UIMC1	AF284753 // UIMC1 // ubiquitin interaction motif containing 1 // 5q35.2 // 51720	AF284753	2.47	AML up vs POOL DONORS
MUC12	NM_001164462 // MUC12 // mucin 12, cell surface associated // 7q22 // 10071 ///	NM_001164462	2.42	AML up vs POOL DONORS
CCL2	NM_002982 // CCL2 // chemokine (C-C motif) ligand 2 // 17q11.2-q12 // 6347 /// E	NM_002982	2.31	AML up vs POOL DONORS
RASD1	NM_016084 // RASD1 // RAS, dexamethasone-induced 1 // 17p11.2 // 51655 /// ENST0	NM_016084	2.23	AML up vs POOL DONORS
LGALS3BP	NM_005567 // LGALS3BP // lectin, galactoside-binding, soluble, 3 binding protein	NM_005567	2.08	AML up vs POOL DONORS
LOC151009	AK095678 // LOC151009 // hypothetical LOC151009 // 2q13 // 151009 /// AK056084 /	AK095678	2.06	AML up vs POOL DONORS
RUNDC2C	NR_002939 // RUNDC2C // RUN domain containing 2C // 16p11.2 // 440352 /// ENST00	NR_002939	2.04	AML up vs POOL DONORS
TP53I11	NM_001076787 // TP53I11 // tumor protein p53 inducible protein 11 // 11p11.2 //	NM_001076787	2.02	AML up vs POOL DONORS
C6orf138	NM_207499 // C6orf138 // chromosome 6 open reading frame 138 // 6p12.3 // 442213	NM_207499	2.01	AML up vs POOL DONORS
EZR	NM_003379 // EZR // ezrin // 6q25.3 // 7430 /// NM_001111077 // EZR // ezrin //	NM_003379	–2.02	AML down vs POOL DONORS
SH3BGR	NM_007341 // SH3BGR // SH3 domain binding glutamic acid-rich protein // 21q22.3	NM_007341	–2.02	AML down vs POOL DONORS
RDH10	NM_172037 // RDH10 // retinol dehydrogenase 10 (all-trans) // 8q21.11 // 157506	NM_172037	–2.03	AML down vs POOL DONORS
IRF6	NM_006147 // IRF6 // interferon regulatory factor 6 // 1q32.3-q41 // 3664 /// EN	NM_006147	–2.05	AML down vs POOL DONORS
FRY	NM_023037 // FRY // furry homolog (Drosophila) // 13q13.1 // 10129 /// ENST00000	NM_023037	–2.05	AML down vs POOL DONORS
RAP1GDS1	NM_001100426 // RAP1GDS1 // RAP1, GTP-GDP dissociation stimulator 1 // 4q23-q25	NM_001100426	–2.09	AML down vs POOL DONORS
PGM5P2	NR_002836 // PGM5P2 // phosphoglucomutase 5 pseudogene 2 // 9q12 // 595135 /// N	NR_002836	–2.11	AML down vs POOL DONORS
MEGF9	NM_001080497 // MEGF9 // multiple EGF-like-domains 9 // 9q32-q33.3 // 1955 /// E	NM_001080497	–2.11	AML down vs POOL DONORS
CYFIP2	NM_001037332 // CYFIP2 // cytoplasmic FMR1 interacting protein 2 // 5q33.3 // 26	NM_001037332	–2.11	AML down vs POOL DONORS
NT5DC3	NM_001031701 // NT5DC3 // 5'-nucleotidase domain containing 3 // 12q22-q23.1 //	NM_001031701	–2.11	AML down vs POOL DONORS
NCAM1	NM_181351 // NCAM1 // neural cell adhesion molecule 1 // 11q23.1 // 4684 /// NM_	NM_181351	–2.17	AML down vs POOL DONORS
ELN	NM_000501 // ELN // elastin // 7q11.23 // 2006 /// NM_001081754 // ELN // elasti	NM_000501	–2.18	AML down vs POOL DONORS
MMP16	ENST00000286614 // MMP16 // matrix metallopeptidase 16 (membrane-inserted) // 8q	ENST00000286614	–2.25	AML down vs POOL DONORS
SDC2	NM_002998 // SDC2 // syndecan 2 // 8q22-q23 // 6383 /// ENST00000302190 // SDC2	NM_002998	–2.26	AML down vs POOL DONORS
CPA4	NM_016352 // CPA4 // carboxypeptidase A4 // 7q32 // 51200 /// NM_001163446 // CP	NM_016352	–2.28	AML down vs POOL DONORS
CD9	NM_001769 // CD9 // CD9 molecule // 12p13.3 // 928 /// ENST00000382518 // CD9 //	NM_001769	–2.30	AML down vs POOL DONORS
DAPK1	NM_004938 // DAPK1 // death-associated protein kinase 1 // 9q34.1 // 1612 /// EN	NM_004938	–2.32	AML down vs POOL DONORS
SLC22A15	NM_018420 // SLC22A15 // solute carrier family 22, member 15 // 1p13.1 // 55356	NM_018420	–2.33	AML down vs POOL DONORS
HAUS6	NM_017645 // HAUS6 // HAUS augmin-like complex, subunit 6 // 9p22.1 // 54801 ///	NM_017645	–2.40	AML down vs POOL DONORS
BMP4	NM_001202 // BMP4 // bone morphogenetic protein 4 // 14q22-q23 // 652 /// NM_130	NM_001202	–2.50	AML down vs POOL DONORS
JAKMIP2	NM_014790 // JAKMIP2 // janus kinase and microtubule interacting protein 2 // 5q	NM_014790	–2.59	AML down vs POOL DONORS
NOV	NM_002514 // NOV // nephroblastoma overexpressed gene // 8q24.1 // 4856 /// ENST	NM_002514	–2.61	AML down vs POOL DONORS
ANO5	NM_213599 // ANO5 // anoctamin 5 // 11p14.3 // 203859 /// NM_001142649 // ANO5 /	NM_213599	–2.64	AML down vs POOL DONORS
SNCA	NM_000345 // SNCA // synuclein, alpha (non A4 component of amyloid precursor) //	NM_000345	–2.66	AML down vs POOL DONORS
PRSS12	NM_003619 // PRSS12 // protease, serine, 12 (neurotrypsin, motopsin) // 4q28.1 /	NM_003619	–2.81	AML down vs POOL DONORS
CPE	NM_001873 // CPE // carboxypeptidase E // 4q32.3 // 1363 /// ENST00000402744 //	NM_001873	–2.82	AML down vs POOL DONORS
GALNT3	NM_004482 // GALNT3 // UDP-N-acetyl-alpha-D-galactosamine:polypeptide N-acetylga	NM_004482	–2.89	AML down vs POOL DONORS
SCN9A	NM_002977 // SCN9A // sodium channel, voltage-gated, type IX, alpha subunit // 2	NM_002977	–2.91	AML down vs POOL DONORS
IGF2BP3	NM_006547 // IGF2BP3 // insulin-like growth factor 2 mRNA binding protein 3 // 7	NM_006547	–2.94	AML down vs POOL DONORS
AHNAK2	NM_138420 // AHNAK2 // AHNAK nucleoprotein 2 // 14q32.33 // 113146 /// ENST00000	NM_138420	–3.06	AML down vs POOL DONORS
SCIN	NM_001112706 // SCIN // scinderin // 7p21.3 // 85477 /// NM_033128 // SCIN // sc	NM_001112706	–3.25	AML down vs POOL DONORS
TM4SF20	NM_024795 // TM4SF20 // transmembrane 4 L six family member 20 // 2q36.3 // 7985	NM_024795	–3.27	AML down vs POOL DONORS
CLDN1	NM_021101 // CLDN1 // claudin 1 // 3q28-q29 // 9076 /// ENST00000295522 // CLDN1	NM_021101	–3.34	AML down vs POOL DONORS
DMKN	NM_001126056 // DMKN // dermokine // 19q13.12 // 93099 /// NM_001190347 // DMKN	NM_001126056	–3.41	AML down vs POOL DONORS
FRMD4B	NM_015123 // FRMD4B // FERM domain containing 4B // 3p14.1 // 23150 /// ENST0000	NM_015123	–3.43	AML down vs POOL DONORS
FBN2	NM_001999 // FBN2 // fibrillin 2 // 5q23-q31 // 2201 /// ENST00000262464 // FBN2	NM_001999	–3.52	AML down vs POOL DONORS
S100A4	NM_019554 // S100A4 // S100 calcium binding protein A4 // 1q21 // 6275 /// NM_00	NM_019554	–3.87	AML down vs POOL DONORS
CMKLR1	NM_001142343 // CMKLR1 // chemokine-like receptor 1 // 12q24.1 // 1240 /// NM_00	NM_001142343	–4.07	AML down vs POOL DONORS
LPCAT2	NM_017839 // LPCAT2 // lysophosphatidylcholine acyltransferase 2 // 16q12.2 // 5	NM_017839	–4.41	AML down vs POOL DONORS
MAMDC2	NM_153267 // MAMDC2 // MAM domain containing 2 // 9q21.12 // 256691 /// ENST0000	NM_153267	–5.02	AML down vs POOL DONORS
FLG	NM_002016 // FLG // filaggrin // 1q21.3 // 2312 /// ENST00000368799 // FLG // fi	NM_002016	–5.33	AML down vs POOL DONORS
SPP1	NM_001040058 // SPP1 // secreted phosphoprotein 1 // 4q22.1 // 6696 /// NM_00058	NM_001040058	–5.95	AML down vs POOL DONORS
C20orf103	NM_012261 // C20orf103 // chromosome 20 open reading frame 103 // 20p12 // 24141	NM_012261	–6.11	AML down vs POOL DONORS
CD24	NM_013230 // CD24 // CD24 molecule // 6q21 // 100133941 /// BC064619 // CD24 //	NM_013230	–7.58	AML down vs POOL DONORS

critical role in hematopoiesis and contribute to bone marrow homeostasis.

Recent studies have highlighted the critical importance of bone marrow MSCs as essential constituents for the HSC niche through production of factors such as CXCL12 and SCF [8,9]. Moreover, studies have provided insights into the role of aberrant microenvironment signaling leading to disease pathology, being MSCs recognized as an essential element of both healthy and leukemic hematopoietic microenvironment [22].

In AML, studies have provided evidence that proliferation, survival and drug resistance could be modulated by MSCs from the BM microenvironment. The signaling between LSCs and MSCs is essential for leukemia survival and disease progression. However, how MSC signaling could affect the leukemogenic process remains unclear [17,23–25].

In this study, we characterized and compared hMSCs from AML patients and healthy donors. Our cultures were derived from AML patients at diagnosis, and when the morphology and proliferation of these cultures were compared to hMSCs from HD, all hMSCs presented normal morphologies and proliferation potentials. One previous study verified the heterogeneous morphology and diverse proliferation capacities among AML cultures compared to hMSCs from HD [26]. These contradictory results could be explained by the differences in patients between the studies; in contrast to our study, this work used AML patients that had been previously treated, and this previous treatment could influence the morphology and proliferation of hMSC-AML.

One fundamental characteristic of MSCs is their potential for adipogenic, chondrogenic and osteogenic differentiation.

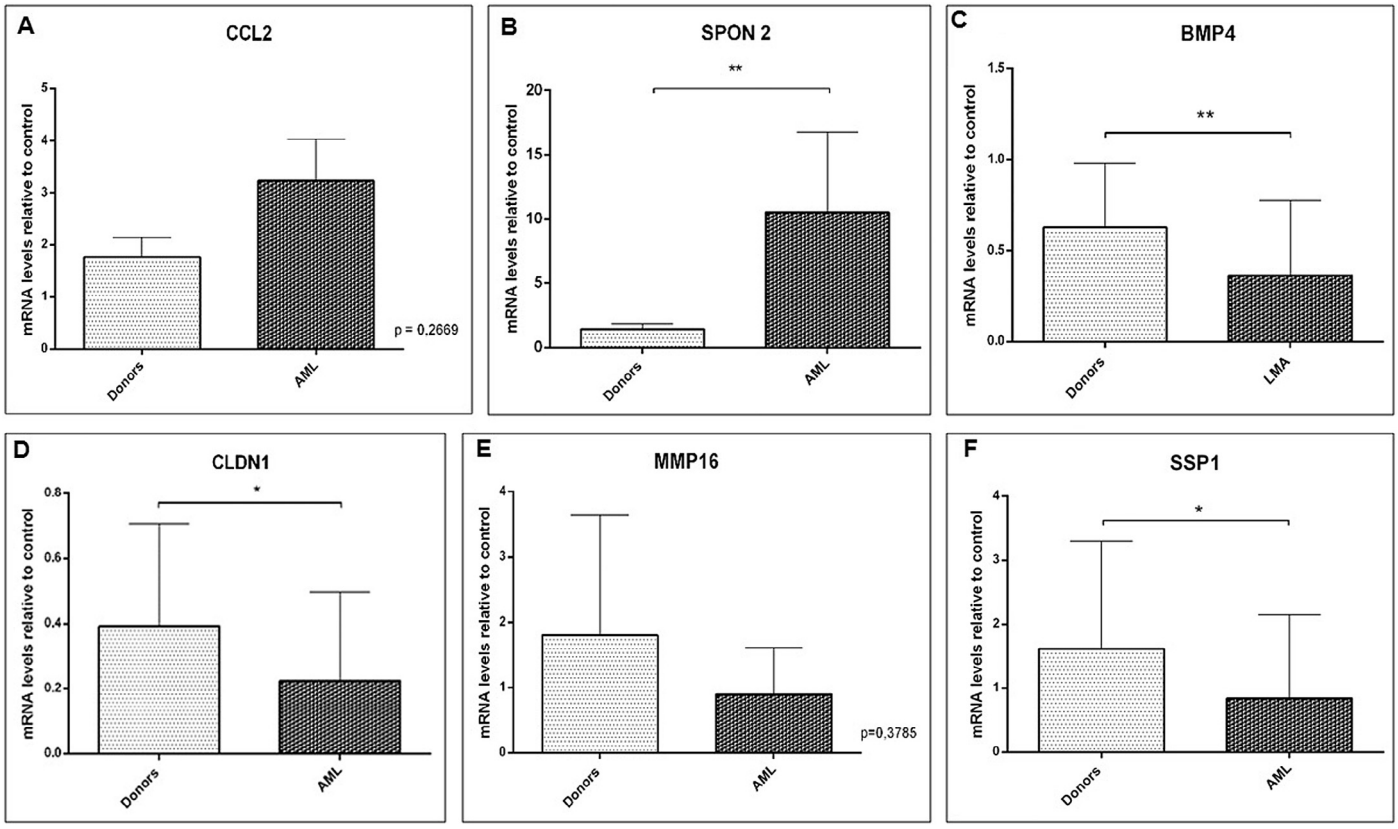

Fig. 4. RT-qPCR to validate the chip array assay results. To confirm the obtained chip array results, RT-qPCR was used to analyze some differentially expressed genes using a larger number of patient samples to determine changes in mRNA expression levels after normalization to *B2M* and *GAPDH* (19 hMSC-AML cultures and 13 hMSC-HD cultures). RT-qPCR analyses of *CCL2* (A) and *SPON2* (B) (overexpressed in AML patients) and *BMP4* (C), *CLDN1* (D), *MMP16* (E) and *SSP1* (F) (downregulated in AML patients) confirmed the chip array assay results and the common molecular signature for hMSCs from all AML patients instead of different molecular signatures for each AML subtype. $^{*}p < 0.05$; $^{**}p < 0.01$.

Osteogenic cells are an important component of the BM microenvironment and have an essential role in regulating normal hematopoiesis [27,28]. Disruption of the osteoblastic compartment results in aberrant hematopoiesis [29]. Thus, unsurprisingly, we found a decrease in osteogenic differentiation potential, which is consistent with the study by Chandran et al. [26] that described a reduced capability of hMSC-AML to support hematopoietic progenitors in the culture. Moreover, studies of hMSCs from patients with myelodysplastic syndrome also found a significantly reduced osteogenic differentiation potential and associated this finding with a reduced ability of MSCs to support CD34+ [30].

To better understand hMSCs-AML, we characterized these cells. The molecular profile of these cells revealed that the 55 differentially expressed genes identified in comparison to hMSCs-HD were able to differentiate hMSCs-AML from hMSCs-HD, suggesting a specific hMSCs-AML molecular signature. After RT-qPCR and unsupervised chip array confirmation, the results confirmed a molecular signature that is capable of distinguishing hMSCs from AML patients from hMSCs from healthy donors. Because we used hMSCs from patient samples at diagnosis, this molecular signature could be an important marker to identify AML patients.

In silico analysis with the 55 differentially expressed genes showed important signaling pathways that could be related to the maintenance and differentiation of HSCs, and alterations in these pathways could influence the leukemogenic process. The osteopontin (SSP1) pathway is important for HSC maintenance. SSP1 regulates the number of HSCs in the bone marrow niche and is an important factor in osteoclast activation [15]. For optimal functioning of the bone marrow, a balance between the number of osteoblasts and osteoclasts is necessary because these cells are responsible for bone formation and resorption.

The other important pathway related to the differentially expressed genes identified in this work is the WNT pathway, which has been related to HSC differentiation. In this study, the WNT pathway could regulate BMP4 expression. BMP4 is a protein secreted into the bone marrow microenvironment, and decreased BMP4 expression may result a disruption of HSC function [20].

Both SSP1 and BMP4, which were identified in our chip array assay, as well as CCL2, are secreted proteins. Thus, if their differential expression could affect the bone marrow microenvironment, their expression should be altered in the bone marrow plasma samples of AML patients. ELISAs using the bone marrow plasma samples from AML patients and from HD confirmed our hypothesis regarding BMP4 and CCL2 proteins. BMP4 and CCL2 were decreased and increased, respectively, in AML bone marrow plasma samples. However, SSP1 protein expression in AML bone marrow plasma samples was identical to that for HD bone marrow plasma samples. This could be due to the expression and secretion of SSP1 by other cell types as osteoblasts, osteoclasts, macrophages, T-cells present in healthy bone marrow environment [31,32].

Otherwise, the decreased expression of BMP4 was unexpected as BMP4 is a member of the TGF-β super family that regulates proliferation, differentiation, cellular survival and cell fate determination. It has been described as a critical component for normal hematopoietic microenvironment that regulates both HSCs number and function and is necessary to maintain functional adult HSCs *in vivo*

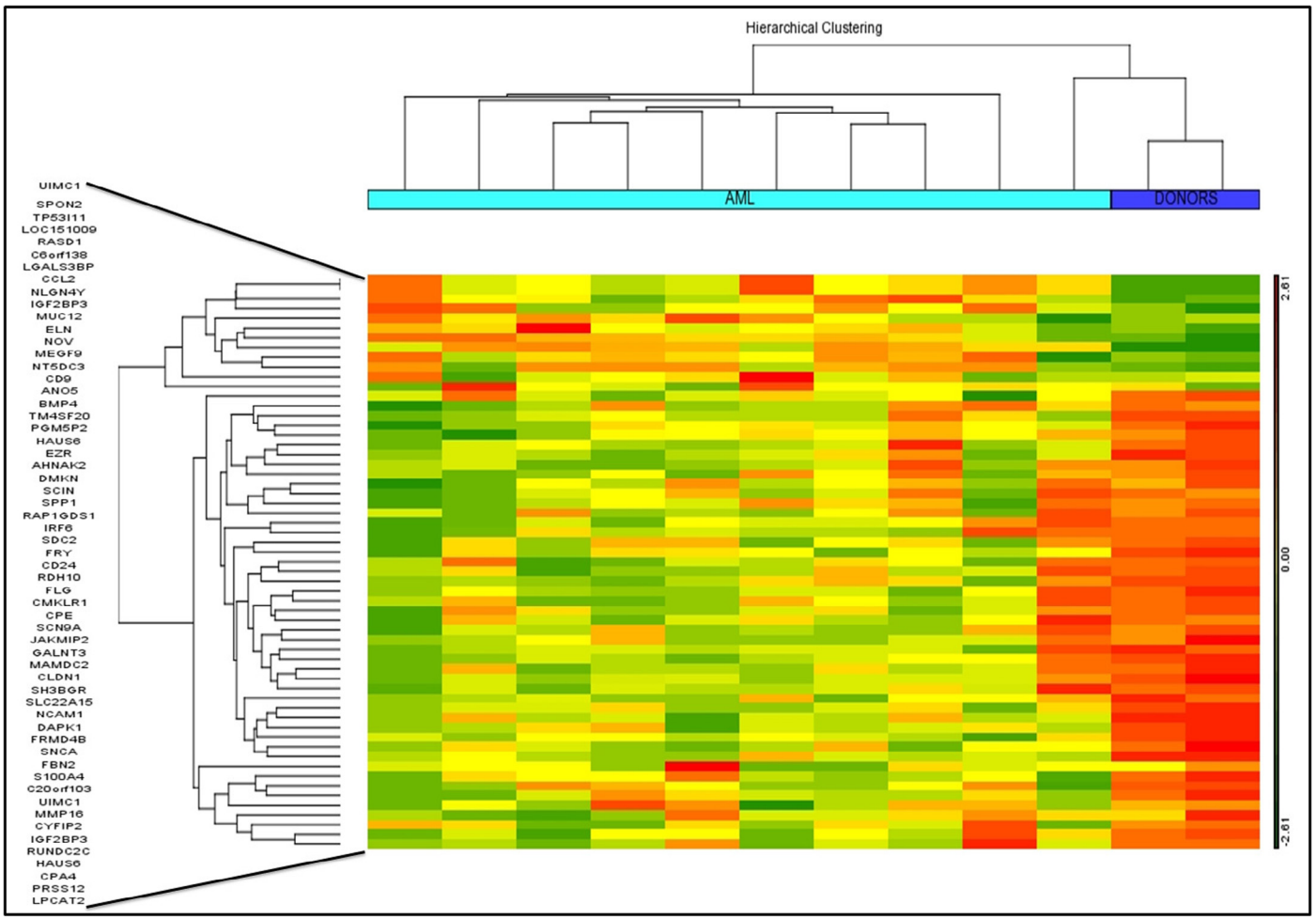

Fig. 5. Unsupervised chip array assay confirmed the molecular signature. Unsupervised analysis of the 55 differentially expressed genes was performed to verify whether the samples could group into clusters that separate hMSCs-AML from hMSCs-HD. The results confirmed that this molecular signature is capable of distinguishing hMSCs from AML patients from hMSCs from HD.

[22]. Deficiency of BMP4 in microenvironment impairs the functional activity of normal HSCs. However, BMP4 decreased expression in both hMSCs from AML patients and AML bone marrow plasma could be related to decreased osteogenesis as BMP signaling pathway is involved in the induction of osteogenic differentiation. So, in the LSCs endosteal niche, it is possible that less cells of the osteogenic lineage are present and that the imbalance between osteoblasts and osteoclast creates a more appropriate niche for LSCs proliferation.

Another secreted protein found deregulated was CCL2. This protein is a cytokine present in the IL-17 signaling pathways that is involved in autoimmune disease, inflammatory processes and the tumor microenvironment [18].

The inflammation in microenvironment has been reported to support tumorigenesis and cancer progression by production of several factors such as growth factors, pro-angiogenic and survival factors [33]. Several works have demonstrated that IL-17, the pro-inflammatory cytokine produced by Th17 cells, can promote tumor growth by enhancing angiogenesis, proliferation, migration and chemoresistance of tumor cells. Also in AML, the expression of IL-17 has been associated with a poor prognostic [34–37].

High levels of CCL2 can contribute to high monocytes migration enhancing the inflammation and can stimulate the production of pro-angiogenic mediators as VEGF [9]. Interestingly, in our chip array results, we found that both VEGFA and VEGFB upregulated in hMSCs from AML patients (1,16- and 1,22-fold change, respectively – data not shown).

Increased levels of CCL2 have been previously reported in serum samples from patients with AML [38], and other studies have found that leukemia cells alter the profiles of cytokines produced by MSCs. These studies demonstrated that enhanced CCL2 expression inhibits normal progenitors but not leukemic cells and improves the survival of these cells [39,40].

In conclusion, the current study suggested a common molecular signature for hMSCs-AML instead of different molecular signatures for each AML subtype. This signature is capable of differentiating between hMSC-AML and hMSC-HD. Moreover, changes in the expression of plasma proteins suggested that hMSCs-AML signaling alterations could be an important factor in the leukemic transformation process. Changes in hMSCs-AML could make the environment permissive for leukemic transformation. hMSCs-AML could collaborate to create a niche more favorable for leukemia cells and promote leukemia maintenance.

Authors' contributions

R.B. and N.C.A.O performed experiments, analyzed the results, made the figures and wrote the paper. B.D.R contributed to proliferation and cell cycle assays. R.B and E.A designed the study.

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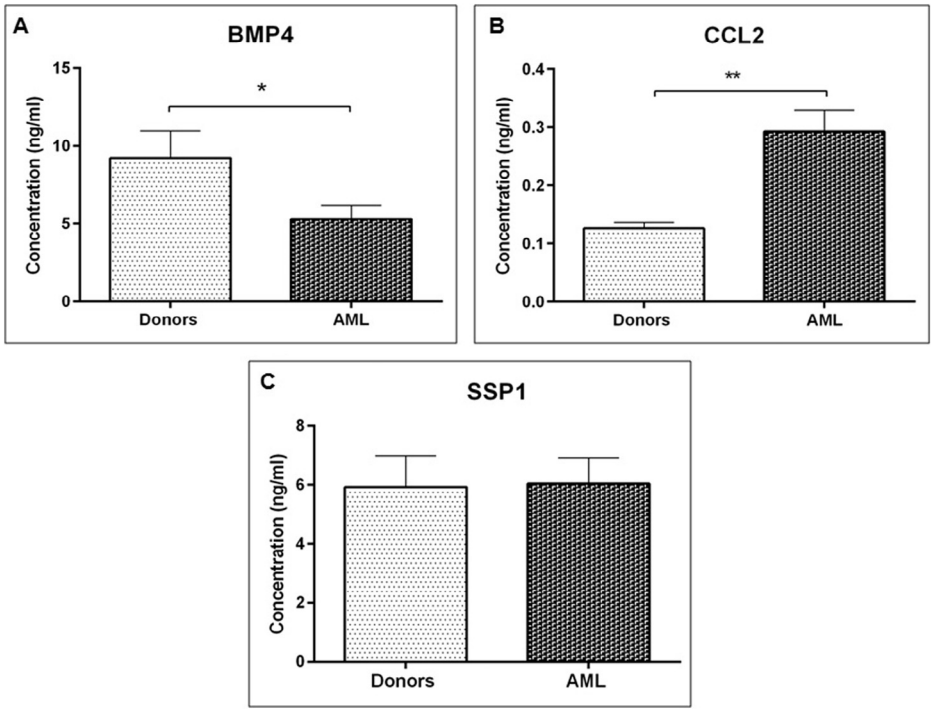

Fig. 6. ELISA with plasma samples. To verify whether the secreted proteins BMP4, SSP1 and CCL2 are differentially expressed in AML patients, we performed ELISA with plasma samples from 33 AML patients and from 11 HD. The results confirmed the decrease in BMP4 (A) and the increase in CCL2 protein (B) levels in AML patient plasma samples. No significant changes were observed for SSP1 protein levels (C). *$p < 0.05$; **$p < 0.01$.

Conflict of interest

The authors have no conflicts of interest to declare.

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